TNF - α Gene Polymorphism is Likely to be a Risk Factor for NASH in Indonesia

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ABSTRACT

Introduction : Non alcoholic steatohepatitis (NASH) is a subset spectrum of NAFLD that can progress toward cirrhosis. Tumor necrosis factor- α (TNF- α) polymorphism play a significant role in liver inflammation and Insulin resistance its pathophysiological hallmark. The association between TNF- α (-238 and -308) polymorphism) and the severity of NAFLD was evaluated. **Method:** A total of 155 subjects (80 NAFLD cases and 75 controls) were included. Liver biopsy was performed for evaluated severity of NAFLD based on NAFLD Activity Score-CRN in all cases. Controls subjects defined was not any liver disease by ultrasound. Plasma TNF– α was measured in all subjects. Polymorphism of` TNF- α promoter gene -308 and -238 were identified using PCR-RFLP confirmed with direct sequencing. **Results:** Liver biopsy established the diagnosis of NASH in 29 cases. There was no association between the incidence of NAFLD with TNF- α polymorphism at the TNF– α -308 or the -238 ($p>0.05$). The prevalence ratio of TNF– α polymorphism -238 was significantly higher for subject with NASH ($p< 0.02$). There was no different prevalence ratio for TNF– α polymorphism -308 ($p>0,05$). We found novel polymorphism of TNF- α -245 T/C in a subject with possible NASH, high plasma TNF- α level (20.27 pg/cc) and very high value of Homeostasis Model of Assessment - Insulin Resistance HOMA-IR (22.73). **Conclusion :** Polymorphism TNF- α -238 is likely to be a risk factor for NASH in Indonesia. The identification of new possible polymorphism of TNF- α -245 requires further study in more samples.

***Keywords**: Polymorphism TNF- α, risk factor, NASH*

INTRODUCTION

Non Alcoholic Steatohepatitis (NASH) is the most severe form of Non Alcoholic Fatty Liver Disease (NAFLD). NASH is clinically a manifestation of chronic liver disease frequently occur in industrial countries and its rate has been increasing in developing countries [1]. Recent epidemiology study (2010) in Asia, Europe, Middle East, North and South America stated the prevalence of NAFLD ranging from 6-35% with median 20%[2]. Hasan Irsan reported that NAFLD was found in 30% of the population in Jakarta based on abdominal ultrasound [3].

Tumor Necrosis Factor (TNF)-α polymorphism has been reported to have effect on the susceptibility of several liver diseases including NASH and more scientific proof showed the involvement of TNF-α in the pathogenesis and progression of liver disease caused by many etiologies [4, 5]. Data from several clinical and experimental studies revealed TNF-α played roles not only in early stage of fatty liver disease but also in transition process to more advanced liver damage. The role of TNF-α in NAFLD is a crossroad of pathogenic pathway. Major difference between patients with simple steatosis and NASH is that serum level of TNF-α is found higher in NASH even though the result did not always reach statistical significance [6].

Two polymorphisms in TNF-α gene promoter have been identified: at position 308 (TNF2 allele)

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and at position 238 (allele TNFA). Studies on the allele TNF-α proved the increased formation and expression of these cytokines compared to the wild type (TNF-1), although the reported data of TNF- α allele was conflicting, but many researchers believed that TNF- α allele increases the release of this cytokine [7].

Luca Valenti (2002) in his research revealed genes that influence the effect of TNF-α , that is polymorphism in gene promoter -238 that correlates well with alcoholic steatohepatitis or NASH(4). Katsutoshi T (2007) reported that serum level of TNF-α was higher in NASH patients than that of steatosis and normal patients. There was no significant deviation in the analysis of TNF-α gene polymorphism between all groups. The number of gene promoter -1031C and -863A in patients with NASH was higher than that of simple steatosis group. Multivariate analysis has proven that polymorphism is an independent factor which differentiate NASH from from simple steatosis. It was concluded that TNF-α polymorphism influences TNF-α production and correlates with the progression of NAFLD [8]. However, data from several studies were conflicting that further study is needed to confirm its correlation.

MATERIAL AND METHODS

A case-control study held in outpatient clinic of dr. Kariadi General Hospital Semarang on January 2009 – December 2011.

Inclusion criteria for case group includes: aged > 14 years old having metabolic syndrome components and proven to have NAFLD based on abdominal ultrasound. Subjects will be excluded if known to have hepatitis A, B, C virus infection (AST and ALT > 5 times higher than normal with positive IgM anti HAV or positive HBsAg or positive anti HCV); autoimmune hepatitis (positive ANA test); alcoholic hepatitis; history of alcohol consumption (> 30 gr/ day for male and > 20 gr/day fpr female); history of taking drugs causing fatty liver (glucocorticoid, estrogen, tamoxifen, amiodarone, metothrexate, valproate, diltiazem).

Inclusion criteria for control group includes no history, and clinical symptoms of liver disease (with normal AST and ALT, negative HbsAg, negative anti HCV) and no liver abnormality found on abdominal ultrasound. Subjects will excluded if known to have liver disease by anamnesis, physical examination and laboratory findings; hepatitis virus infection (A,B,C); autoimmune hepatitits, alcoholic hepatitis; history of alcohol consumption; and history of taking drugs causing fatty liver.

All subjects underwent these following physical and laboratory examinations: blood pressure, body weight, height, body mass index, waist circumference, liver enzymes (AST, ALT), lipid profile (HDL, total cholesterole, triglyceride) and fasting blood glucose. Serum level of TNF-α was tested in all subjects of case and control groups. Liver biopsy was performed only in NAFLD subjects for classification of severity by Kleiner et al; NASH Clinical research network scoring system (NAFLD activity score =NAS). NASH was defined if NAS > 5, Possible NASH if NAS 3-4 and Simple steatosis if NAS < 3 consecutively. TNF-α polymorphism in gene promoter -238 and -308 was tested using Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) and confirmed by direct sequencing.

RESULTS

Basic Characteristics

During study period, 150 subjects were enrolled and divided into 2 groups. Case group (n=75) consisted of NAFLD patients and control group (n=75) consisted of healthy subejcts without fatty liver. The clinical characteristics of all subjects in both groups are shown in table 1.

Table 1. Clinical characteristics, serum level of TNF-α and gene promoter polymorphism of subjects in case and control group

Characteristic	Group		p
	Case (n=75)	Control (n=75)	
Sex α			
Male	45 (55,6%)	36 (44,4%)	
Female	30 (43,5%)	39 (56,5%)	< 0,100*
Age (year)£	44,7 ±10,28 (23,0 - 74,0)	44,3 ± 9,98 (20,0 - 72,0)	< 0,800§
Male age (year)£	42,6 ±10,36 (25,0 - 74,0)	42,0 ± 11,04 (20,0 - 72,0)	< 0,800§
Female age (year)£	48,0 ± 9,42 (25,0 74,0)	46,5 ± 8,47 (26,0 - 63,0)	< 0,500§
Body weight (kg)$	77,0 (54,0 - 115,0)	56,0 (41,0 - 74,0)	< 0,001¶
Height (cm)£	162,1±8,37 (140 – 178)	161,1±6,31 (147,0 - 179,0)	< 0,400§
Body mass index $	29,0 (22,0 - 47,4)	22,5 (17,3 - 27,5)	< 0,001¶
Waist circumference (cm)$	99,0 (77,0 - 134,0)	80,0 (64,0 - 98,0)	< 0,001¶
TNF-α (pg/ml) $	7,98 (0,00-332,56)	2,24 (0,04-77,65)	<0,001¶
TNF-α (pg/ml) >3,645	58 (76,3%)	18 (23,7%)	
□3,645	17 (23,0%)	57 (77,0%)	<0,001*
TNF-α gene promoter polymorphism			
Region-238			
G/G	72(96,0%)	73 (97,3%)	
A/G	3 (4,0%)	2 (2,7%)	
A/A	0 (0,0%)	0 (0,0%)	
Frequency alel A (%)	2,0	1,3	0,700□
Region-308			
G/G	74(98,7%)	72(96,0%)	
A/G	1(1,3%)	3(4,0%)	0,600□
A/A	0(0,0)	0(0,0)	
Frequency alele A(%)	0,7	2	

£ Mean ± standard deviation (minimum-maximum); $ Median (minimum – maximum)
*x^2 test; §Independent t-test; ¶Mann-Whitney test, zFisher-exact test, Frequency of alele A=(frequency of alele A/total alele) x 100%

Serum level of TNF-α

Serum level of TNF-α of subjects in case and control groups is shown in table 1. The result shows that mean serum level of TNF- α in case group was significantly higher than that of control group (p<0,001).

Cut-off level of TNF- α to diagnose NAFLD

The result of receiver operating characteristics (ROC) analysis for TNF- α to determine the incidence of NAFLD. Area under the ROC curve of serum level of TNF- α to determine the incidence of NAFLD was 0,80 (95% CI=0,73 s/d 0,87) and it was statistically significant (p<0,001). ROC analysis also demonstrated cutoff point for serum level of TNF- α to determine the incidence of NAFLD was 3,645 ng/ml. Based on the cutoff point, further analysis was done. The subjects with serum level of TNF- α > 3,645 pg/ml were in the case group (76,3%), and on the opposite, subjects with TNF- α ≤ 3,645 pg/ml were mostly in the control group (77%). Statistical analysis confirmed that the significant correlation between serum level of TNF- α and the incidence of NAFLD.

TNF- α gene promoter 238 dan -308 polymorphism in NAFLD

The results of PCR-RFLP investigating TNF-α gene promoter -308 G/A and -238 G/A polymorphism are visulized in figure 1a and Ib

Results of liver biopsy.

Liver biopsy is a gold standard for the diagnosis of NAFLD severity. Most of the subjects in this case group of severity were included in the Possible NASH in 38 subjects (50.67%), then NASH in 29 subjects (38.67%) and at least the Simple Steatosis in 8 subjects (10.67 %).

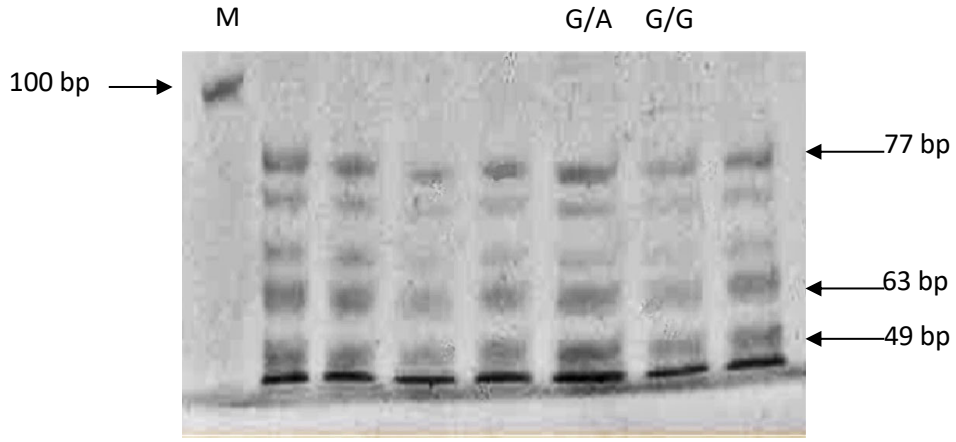
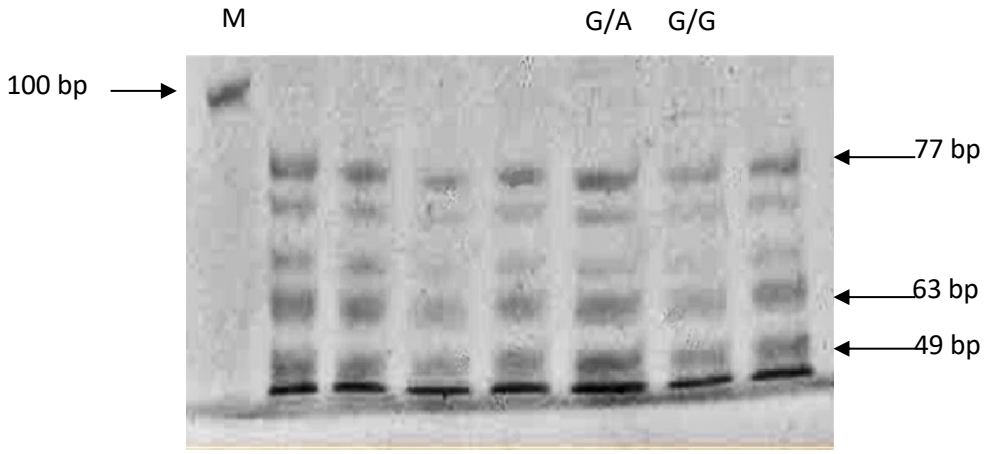


Figure 1b . Agarose gel visualization for TNF-α gene promoter -238 (PCR-RFLP) polymorphism

Showing agarose gel as the result of PCR-RFLP TNF-α regio promoter -238 polymorphism. Size of product after RFLwas 77 bp, 63 bp, 49 bp and 21 bp for -238 G/A polymorphism. Size of *wildtype* -238 G/G was 77, 70, 63, dan 49 bp. Product sized 70 bp and 21 bp were not visualized on agarose gel. No allele 2 (-238 A/A) was found



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TNF- α gene promoter -238 and -308 polymorphisms

TNF- α polymorphism test using PCR-RFLP method was confirmed by direct sequencing due to very little polymorphism found by the PCR-RFLP in Indonesian population, or there was possibly other kind of univestigated polymorphism. Distribution of TNF- α polymorphism located in gene promoter -238 and -308 is listed in table 1. In table 1, the genotype of TNF- α promoter -238 mostly seen in both case and control group was G/G. TNF- α -gene 238A/G polymorphism was found in 5 subjects (3 in case group and 2 in control group). TNF- α -gene 238A/A polymorphism was not found. The frequency of allele A TNF- α -gene 238 polymorphism in case group was higher than that of control group. Statistical analysis shows no significant difference in the incidence of TNF- α -gene 238A/G polymorphism between case and control group (p=0,7).

In table1, the genotype of TNF- α promoter -308 mostly seen in both case and control groups was G/G. TNF- α -gene 308A/G polymorphism was found in 4 subjects (1 in case group and 3 in control group). Uncommon allel in control group was found more frequent in control group compared to case group (typical for Javanese-Indonesian population). TNF- α -gene 308A/A polymorphism was not found. The frequency of allele A TNF- α -gene 308 polymorphism in control group was higher than that of case group. Statistical analysis shows no significant difference in the incidence of TNF- α -gene 308A/G polymorphism between case and control group (p=0,6).

Serum level of TNF- α based on TNF- α - gene 238 and -308 polymorphisms

Table 2 below shows serum level of TNF- α based on TNF- α - gene 238 and -308 polymorphisms.

Table 2. Serum level TNF- α based on genotype of TNF- α -238 and 308

Promoter region	Allel TNF- α		p¶
	GG	AG	
- TNF- α -238	3,56 (0,00 - 332,56)	22,42 (3,56 - 107,87)	0,020
- TNF- α -308	3,89 (0,00 - 332,56)	2,36 (1,51 - 31,93)	0,600

§ Median (minimum – maximum)

¶ Mann-Whitney test

Data on table 2 shows that serum level of TNF-α in subjects with TNF- α gene -238A/G polymorphism (n=5) has higher level of TNF- α than that of without polymorphism (n=145) with significant difference (p=0,02). Serum level of TNF- α in subjects without TNF- α gene -308 polymorphism (n=146) has higher level of TNF- α than that of with TNF- α -308A/G polymorphism (n=4) but the difference was not statistically significant (p=0,6).

Distribution of TNF- α gene promoter -238 and -308 polymorphisms

TNF- α -gene 238 polymorphisms was found in 5 subjects (3 NASH and 2 central obesity (-)). There was no difference in TNF- α -gene 238 polymorphisms between groups (p = 0,1). TNF- α -gene 308 polymorphisms was found in 4 subjects (1 NASH, 2 central obesity (+), 1 central obesity (-)). There was no difference in TNF-α -gene 308 polymorphisms between groups (p = 0,1). DNA sequencing of TNF- α gene -308 G/A, -238 G/A, -245 T/C and wild type is picturized in figure 2.

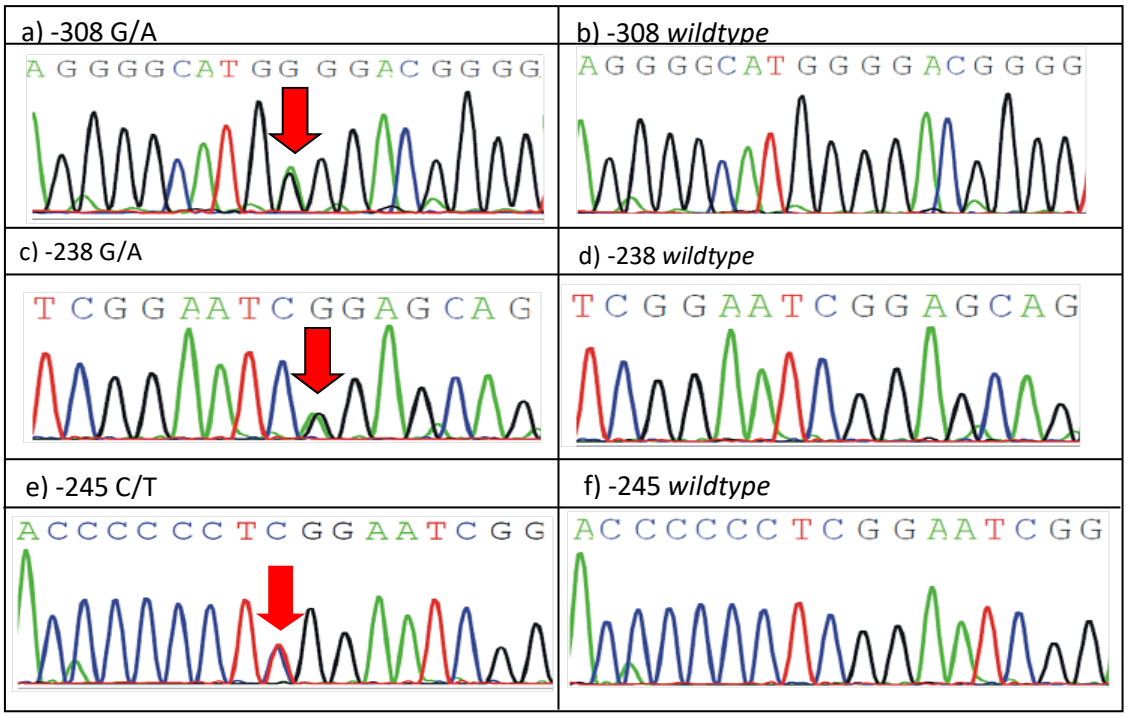

Figure 2. DNA sequencing chromatogram of gene polymorphism and wild type of TNF-α -308, -238, and -245

As seen in figure 2, sequencing chromatogram shows the presence of polymorphism (red band) (overlapping band/ heterozygote) in TNF- α –gene promoter -308 G/A (a), TNF- α -gene promoter -238 G/A (c), and TNF- α -gene promoter -245 C/T (e). Figure b,d and f consecutively are the wild type of TNF- α -gene promoter -308, -238 dan -245.

Table 3. Risk factor of NAFLD

Risk factor	Group		Odd Ratio (95% confidence interval)	p*
	Case n=75 n (%)	Control n=75 n (%)		
TNF- α (pg/mL)				
> 3,645	58 (76,3%)	18 (23,7%)	10,8 (5,1 s/d 23,0)	< 0,001
α 3,645	17 (23,0%)	57 (77,0%)		
TNF- α -gene 238A/G Polymorphism				
Present	3 (60%)	2 (40,0%)	1,5 (0,2 s/d 9,4)	0,700
Absent	72 (49,7%)	73 (50,3%)		
TNF- α -gene 308A/G Polymorphism				
Present	1 (25,0%)	3 (75,0%)	0,3 (0,03 s/d 3,2)	0,600
Absent	74 (50,7%)	72 (49,3%)		

* α ^{2} test

TNF-α and TNF-α gene polymorphism as risk factor of NAFLD

Analysis of TNF-α level TNF-α gene polymorphism as risk factor of NAFLD can be seen in table 3 Subjects with serum level of TNF-α > 3,645 pg/mL are 10,8 times more likely to develop NAFLD compared to those whose level of TNF- α 3,645 pg/mL. As listed in table 3, TNF-α > 3,645 pg/mL is a risk factor for developing NAFLD (p < 0,001). On the other hand, TNF- α gene polymorphism was not proven to be risk factor of NAFLD.

TNF-α and TNF-α gene polymorphism as risk factor of NASH

Analysis for risk factor of NASH is listed on table 4. Non NASH group consists of combination of subjects with suspected NASH, simple steatosis, central obesity (+) and central obesity (-).

Table 4 . Analysis for risk factor of severity of NAFLD

Risk factor	Group		Prevalence ratio (95% confidence interval)	p
	NASH n=29 n (%)	Non NASH n=126 n (%)		
TNF- α (pg/mL)				
> 9,41	18 (39,1%)	28 (60,9%)	3,9 (2,0 s/d 7,5)	< 0,001*
α 9,41	11 (10,1%)	98 (89,9%)		
TNF- α -gene 238A/G Polymorphism				
Present	3 (60,0%)	2 (40,0%)	3,5 (1,6 s/d 7,7)	0,020¥
Absent	26 (17,3%)	124 (82,7%)		
TNF- α -gene 308A/G Polymorphism				
Present	1 (25,0%)	3 (75,0%)	1,3 (0,2 s/d 7,6)	0,700¥
Absent	28 (18,5%)	123 (81,5%)		

* α ^{2} test n=75 healthy control by ultrasound, n=80 cases by liver biopsy (Nafld activity score)
¥ Fisher-exact test

TNF- α -gene 238A/G polymorphism has prevalence ratio 3,5 (1,6 s/d 7,7) which means subjects having TNF-α -gene 238A/G polymorphism are 3,5 times more likely to develop NASH compared to those without TNF-α -gene 238A/G polymorphism. Based on 95% confidence interval, TNF- α -gene 238A/G polymorphism was concluded as risk factor of NASH. TNF- α -gene 308A/G polymorphism was not considered as risk factor of NASH based on the analysis despite the prevalence ratio (4, 9)

DISCUSSION

This study was first performed in Indonesia nonalcoholic fatty liver disease patients linked to the level TNF- α, severity and polymorphism gene.

Tumor necrosis factor- α is well-known as major cytokine that plays important role in the occurance of liver damage, including fatty and inflammatory liver disease (steatohepatitis) [10, 11]. Studies investigating about TNF- α gene polymorphism in NAFLD have been showing conflicting results. Several studies reporting the correlation of TNF- α polymorphism and susceptibility of NASH[11-14] yet there were also other studies not supporting the theory [8, 15]. This study focused on 2 polymorphisms located in region -308 dan -238

The findings in our study were inconsistent with previous studies by Valenti et al (2002) and Murillo et al (2011) in a population of Mexican and Italian that reported gene polymorphism [9, 12, 13, 16]. This study reported TNF-α -238 as risk factor of NASH /severity of NAFLD, whereas TNF-α gene polymorphism-308 was not a risk factor of NAFLD. In contrast to previous research results by Aller et al (2010) in Spanish population TNF-α gene polymorphism -308 was found to be associated with histopathologic changes of the liver [13]. Tokushige (2007) reported in the Japanese population polymorphism associated with TNF-α increased blood levels of TNF-α [8]. Additionally Tokushige also reported TNF-α gene polymorphism positions -1031, -863 instead of the -857, -308 and -238 associated with progression of NAFLD (incidence of NASH)[12]. The results of the meta-analysis by Wang et al (2012) in 7 case-control study TNF-α gene polymorphisms and 8 case-control studies concluded; TNF-α gene polymorphism -238 was a risk factor NAFLD, whereas gene polymorphism TNF-α-308 was not a risk factors NAFLD [17]. Murillo at al (2011) also reported gene polymorphism TNF-α -238 associated with the onset of sensitivity NASH [12].

In accordance to the previous studies, we found that serum level of TNF-α is significantly higher in subjects with TNF-α gene -238 polymorphism compared to those without TNF-α gene -238 polymorphism. There was no difference in serum level of TNF-α between subjects with and without TNF-α gene -308 polymorphism. TNF-α gene -238 polymorphism is not only related to the increase of serum level of TNF-α, but also to the occurence of insulin resistance [8]. TNF-α has been reported to cause trauma and apoptosis of liver tissue, play role in netrophil chemotactic, activation of stellate cells of the liver and related to insulin resistance locally in liver tissue or systemically [18]. Crespo et all (2001) reported significant increase of TNF-α expression and receptors in liver and increase of TNF-α expression in adipose tissue in NAFLD patients with obesity [19].

Ethnical variety determines the different roles of metabolic and sosio-demography risk factors in NASH [20], therefore further study involving different ethnicity is needed to confirm the pathogenesis. This study has shown that TNF-α polymorphism in Indonesian population (specifically Javanese-dominant) is different from Kaukasian population. It is suggested to conduct a larger study (multicenter) to prove that TNF-α gene -238 A/G polymorphism is a risk factor of severity of NAFLD (risk factor of incidence of NASH) considering the significantly high level of TNF-α was found in subjects with TNF-α gene -238 polymorphism.

This study also found one subject with 2 (doubles) polymorphisms (haplotypes) that have the highest severity of NAFLD (NAS score = 7). In addition, it was also found in this study 1 subject with novel polymorphism, that is TNF- α - 245 T/C with clinical characteristics showing high level of insulin resistance and very low level of adiponectin (1401 ng/cc). Further study with a larger sample is suggested to explain better the consistency of the relationship.

The limitation of this study were tools for determining the presence of fatty liver in case group and the healthy control were not the same, liver biopsies were performed only in the case group, while the healthy control group with ultrasonography, this potentially arising the measurement bias.

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